



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the Application of:

JONATHAN EDWARD LIGHTNER
JOHN JOSEPH OKULEY

CASE NO.: BB-1043-A

SERIAL NO.: 08/256,047

GROUP ART UNIT: 1803

FILED: OCTOBER 7, 1994

EXAMINER: MCELWAIN

COPY OF PAPERS
ORIGINALLY FILED

FOR: GENES FOR MICROSOMAL DELTA-12
FATTY ACID DESATURASES AND
RELATED ENZYMES FROM PLANTS

Date: JANUARY 30, 1998

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

DECLARATION OF DR. ANTHONY JOHN KINNEY UNDER 37 CFR 1.132

I, Anthony John Kinney declare as follows:

1. I am a citizen of the United Kingdom and am a permanent resident of the United States of America, residing in Wilmington, Delaware.

2. I received a B.Sc. in biology from the University of Sussex in 1980 and a D. Phil. in biochemistry and cell biology from Oxford University in 1985.

3. I served as a research fellow in the Department of Food Science at Rutgers University, New Brunswick, N.J. 9/87-5/89.

4. I have been employed at E. I. du Pont de Nemours and Company (DuPont) since June, 1989 and presently work as a principal investigator for DuPont's agricultural products and am presently working on expression of storage oil genes.

5. I have authored in excess of fifteen refereed articles in the field of biochemistry.

6. I have reviewed the above-identified case, and the Official Action for the subject case dated October 30, 1997. I understand that this declaration is being submitted to address the issues of the pending claims under 35 USC §112 first paragraph or second paragraph. Enclosed herewith are a number of amino acid sequence alignments and phylogenetic tree topologies of mammalian plant (18:1 acyl-ACP) delta-12 desaturases or acyl-ACP 12-hydroxylases which were discussed with the Examiner at the interview held on December 9,

I HEREBY CERTIFY THAT THIS PAPER IS BEING DEPOSITED WITH THE UNITED STATES
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ON THIS DATE

1/30/98
Date

Anthony J. Kinney
Signature

1997. All of these figures were prepared using the Megalign program from DNASTar, Inc., applying the comparison method of Hein (1990, *Meth. Enz.* **183**:626-546).

The subject case discloses nucleic acid fragments encoding several plant C18:1 acyl-ACP delta-12 desaturases and a plant C18:1 12-hydroxylase which can be characterized as a delta-12 desaturase related enzyme. These nucleic acids encode proteins that form a distinct class of enzymes with regard to their amino acid sequence conservation.

Attachment I presents an alignment of the polypeptides set forth in SEQ ID NOS:2, 4, 6, 8, and 12; these are the predicted gene products from the nucleotide sequences set forth in SEQ ID NOS:1, 3, 5, 7, and 11. The shaded residues in the alignment indicate those residues that are conserved when compared with the *Arabidopsis* polypeptide (SEQ ID NO:2).

Attachment II is a phylogenetic tree depicting the calculated evolutionary/structural relationship of the sequences aligned in Attachment I. These figures clearly depict the close evolutionary/structural relationship among these proteins. Furthermore, it is also apparent that the 12-hydroxylase from castor is a member of this class of proteins. Within these alignments it can be seen that the delta-12 desaturase from soybean is more closely related to the hydroxylase from castor than it is to the other delta 12-desaturases disclosed in the subject specification. The sequences disclosed in the instant specification are also very closely conserved with other plant delta-12 desaturases.

Attachment III presents the amino acid sequence alignment of the previously mentioned polypeptides with those of other desaturases that have appeared in the public domain since the filing of the subject case. The additional sequences are for the proteins from *Brassica juncea*, parsley, potato, sunflower, and peanut (GenBank or EMBL accession numbers X91139, U86072, X92847, U91341, and AF030319, respectively). The corresponding phylogenetic tree for this expanded group of protein sequences is found (plotted on a logarithmic scale) in Attachment IV. These comparisons show clearly the relationship between these sequences, and convey graphically the expectation that any one of nucleic acid sequences set forth in SEQ ID NO:1, 3, 5, 7, or 11, may be used as a hybridization probe for genes encoding other plant delta-12 desaturases or desaturase related enzymes, such as 12-hydroxylases. This has been confirmed in my laboratory.

Delta-12 desaturase or 12-hydroxylase protein sequences are structurally distinguishable from those of the next most closely related class, the delta-15 desaturases. Attachment V presents an amino acid sequence alignment of the previously described 12-

desaturases with the sequences of plant delta-15 desaturases that were disclosed in WO 93/11245 (now pending USSN 08/244205). As suggested by the data within the specification for the subject case, it is relatively easy to identify delta 12-desaturases on the basis of their similarity, and moreover to distinguish them from the delta-15 desaturases due to their dissimilarity. The shading in this figure indicates those residue that are conserved relative to the delta-12 desaturase of *Arabidopsis*. Alignment of the 12-desaturases indicates that there is approximately 50% strict conservation or identity of amino acid residues across these proteins, however, they share only 30%-35% identity with any of the delta-15 desaturases. The phylogenetic tree depiction (Attachment VI) shows quite clearly that the two classes do not overlap. Rather, their members are separated by a highly statistically significant divergence, as is indicated by the scale along the bottom of the figure. Further support for this distinction comes from failed experimental attempts to use a gene encoding a plant delta-15 desaturase as a hybridization probe to identify and isolate genes that encode plant delta-12 desaturases.

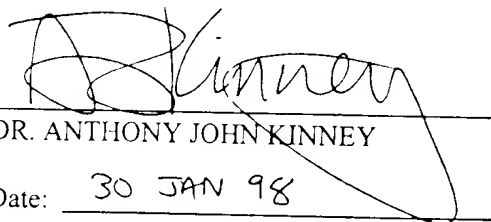
The delta-12 desaturases are members of a group of proteins that catalyze various oxidations of C18:1 fatty acyl groups at the C12-C13 bond. That is, other activities are known which catalyze mechanistically similar oxidations at this position, but do not necessarily produce a double bond in the product. However, it is not possible to say that these represent different classes of structures.

Disclosed in the subject case is the cDNA sequence from *Ricinus communis* (castor bean) encoding a 12-hydroxylase, which is a desaturase-related enzyme. The amino acid sequence of this protein is seen to fall cleanly within the "delta-12 desaturase" group when analyzed by a comparison method such as that of Hein (1990). Attachment V and VI also include within their presentations the placement of the hydroxylase from *Lesquerella fendleri* (WO 97/30582, SEQ ID NO:4). What is striking about this comparison is that the *Lesquerella* hydroxylase and the *Arabidopsis* delta-12 desaturase are more closely related to each other than they are to any other hydroxylase or desaturase included in the analysis. Not only are the proteins indistinguishable on the basis of their sequence, but it is now known that it may not be possible to distinguish them with regard to their function. Braun et al. recently described results of experiments which sought to identify amino acid residues that disposed a protein to being either a desaturase or a hydroxylase (Braun et al., in: *Physiology, Biochemistry, and Molecular Biology of Plant Lipids*, Williams, J.P. et al. eds., Kluwer Academic Publishers, Dordrecht, 1997, pages 342-344; a copy of which is enclosed herewith). During the course of their work, the authors expressed the "hydroxylase" from *Lesquerella* in yeast under the control of an inducible promoter, and found that when

compared to the uninduced control, the induced transformant produced both the C18:2 fatty acid, linoleic acid, and the 12-hydroxy form of oleic acid, ricinoleic acid. As it is known that yeast normally does not make detectable amounts of either of these oleic acid derivatives, it follows that the single protein from *Lesquerella* is responsible for the production of both products. Accordingly, it is both a "desaturase" and a "hydroxylase." These researchers then showed that mutation of only six amino acids (out of a total of 384 residues) was sufficient to convert this to a protein with properties closer to those of a "pure" desaturase. These results are depicted graphically in Figure 1 of the reference. Thus, it is apparent that this structural and functional class of proteins certainly includes more than enzymes that catalyze desaturation.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,



DR. ANTHONY JOHN KINNEY

Date: 30 JAN 98



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Attachment I

F T V G Q L K F A I P P H C F E R S I L R S F S Y V V Y D L I I A S C F Y Y V A Majority

TTYFPLLFSPLSYXAWPLYWACQGCVLTGVWVIAHECGHH Majority

AF APTV GWT F DV V G L I L H S F L L V E Y F S W R Y S H R R H H S N T G S Majority

LEND EVFVPKXKSAISWYSKYL - NNPLGFVVMLTVQLTLG Majority

WFLYLA FNVS GR EYGG - FACHFL EYAI IYNDREERLQ IYV : Majority

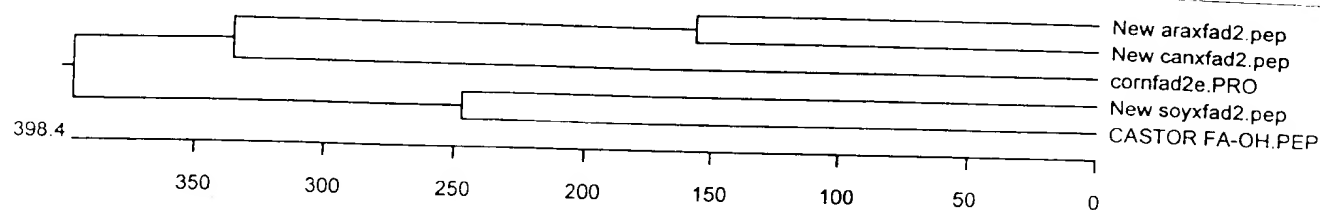
	210	220	230	240	
1	WPMYLAENFVSGRPYDS	FASHYHPYAP	PIYSNFERLLIYVS	NEW	glycylad3 pep
2	WPLYLAENFVSGRPYDS	FASHYHPYAP	PIYSNFERLLIYVS	NEW	glycylad3 pep
3	WPLYLAENFVSGRPYDS	FASHYHPYAP	PIYSNFERLLIYVS	NEW	glycylad3 pep
4	WPLYLAENFVSGRPYDS	FASHYHPYAP	PIYSNFERLLIYVS	NEW	glycylad3 pep
5	WPLYLAENFVSGRPYDS	FASHYHPYAP	PIYSNFERLLIYVS	NEW	glycylad3 pep

DAGILAVTFGLYRYAAACGVAVWVVCVYGVPLLIIVNGFL.L																														Majority											
250										260										270										280											
125	D	V	A	L	F	S	V	T	Y	S	L	Y	R	V	A	T	L	K	G	L	V	W	L	L	C	V	Y	G	V	P	L	L	I	V	N	G	F	L	V	T	New soyxfad2.pep
133	D	L	G	I	F	A	T	T	F	V	L	Y	Q	A	T	M	A	K	G	L	A	W	V	M	R	I	Y	G	V	P	L	L	I	V	N	C	F	L	V	M	CASTOR FA-OH.PEP
129	D	A	G	I	L	A	V	C	F	G	L	Y	R	Y	A	A	A	Q	G	M	A	S	M	T	C	L	Y	C	V	P	L	L	I	V	N	A	F	L	V	L	New araxfad2.pep
129	D	A	G	I	L	A	V	C	Y	G	L	L	I	Y	A	A	V	O	G	V	A	S	M	V	C	F	L	R	V	P	L	L	I	V	N	G	F	L	V	L	New canxfad2.pep
136	D	A	G	V	V	A	V	A	F	G	L	Y	F	L	A	A	A	F	G	V	W	V	V	R	V	Y	A	V	P	L	L	I	V	N	A	W	L	V	L	cornfad2e.PRO	
ITYLQHTHPSLPHYDSSSEWDWLRGALATVDRDYGILNKFV																														Majority											
290										300										310										320											
165	I	T	Y	L	Q	H	T	H	F	A	L	P	H	Y	D	S	S	E	W	D	W	L	R	G	A	L	A	T	M	D	R	D	Y	G	I	L	N	K	V	F	New soyxfad2.pep
173	I	T	Y	L	Q	H	T	H	P	A	I	P	F	Y	G	S	S	E	W	D	W	L	R	G	A	M	V	T	V	D	R	D	Y	G	V	L	N	K	V	F	CASTOR FA-OH.PEP
169	I	T	Y	L	Q	H	T	H	P	S	L	P	H	Y	D	S	S	E	W	D	W	L	R	G	A	L	A	T	V	D	R	D	Y	G	I	L	N	K	V	F	New araxfad2.pep
169	I	T	Y	L	Q	H	T	H	P	S	L	P	H	Y	D	S	S	E	W	D	W	L	R	G	A	L	A	T	V	D	R	D	Y	G	I	L	N	Q	G	F	New canxfad2.pep
176	I	T	Y	L	Q	H	T	H	P	S	L	P	H	Y	D	S	S	E	W	D	W	L	R	G	A	L	A	T	M	D	R	D	Y	G	I	L	N	R	V	F	cornfad2e.PRO
HNIITDTHVAHHLFSTMPHYHAMEATKAIKPILGEYYQFDG																														Majority											
330										340										350										360											
305	H	N	I	T	D	T	H	V	A	H	H	L	F	S	T	M	P	H	Y	H	A	M	E	A	T	N	A	I	K	P	I	L	G	E	Y	Y	Q	F	D	D	New soyxfad2.pep
313	H	N	I	A	D	T	H	V	A	H	H	L	F	A	T	V	P	H	Y	H	A	M	E	A	T	K	A	I	K	P	I	M	G	E	Y	Y	R	Y	D	G	CASTOR FA-OH.PEP
309	H	N	I	T	D	T	H	V	A	H	H	L	F	S	T	M	P	H	Y	N	A	M	E	A	T	K	A	I	R	P	I	L	G	D	Y	Y	Q	F	D	G	New araxfad2.pep
309	H	N	I	T	D	T	H	E	A	H	H	L	F	S	T	M	P	H	Y	H	A	M	E	A	T	K	A	I	K	P	I	L	G	E	Y	Y	Q	F	D	G	New canxfad2.pep
316	H	N	I	T	D	T	H	V	A	H	H	L	F	S	T	M	P	H	Y	H	A	M	E	A	T	K	A	I	R	P	I	L	G	D	Y	Y	H	F	D	P	cornfad2e.PRO
TPVYKALWREAKECITYVEPDEGGDKKG'. WYNNKY																														Majority											
370										380										390																					
401	T	P	V	Y	K	A	L	W	R	E	A	K	E	C	I	Y	V	E	P	D	E	G	G	D	K	K	G	V	Y	W	Y	N	N	K	Y	New soyxfad2.pep					
403	T	P	F	Y	K	A	L	W	R	E	A	K	E	C	L	F	V	E	P	D	E	G	A	P	T	Q	G	V	F	W	Y	R	N	K	Y	CASTOR FA-OH.PEP					
409	T	P	W	Y	V	A	M	Y	R	E	A	K	E	C	I	Y	V	E	P	D	R	E	G	D	K	K	G	V	Y	W	Y	N	N	K	L	New araxfad2.pep					
409	T	P	V	V	K	A	M	W	R	E	A	K	E	C	I	Y	V	E	P	D	R	Q	G	E	K	K	G	V	F	W	Y	N	N			New canxfad2.pep					
405	T	P	V	A	K	A	T	W	R	E	A	K	E	C	I	Y	V	E	R	-	-	-	D	R	K	G	V	F	W	Y	N	N	K	F	cornfad2e.PRO						

Decorations: Decoration #2: Shade (with bright turquoise at 40% fill) residues that match New araxfad2.pep exactly.

New soyxfad2.pep = soybean delta-12 desaturase
CASTOR FA-OH.PEP = castor 12-hydroxylase
New araxfad2.pep = Arabidopsis delta-12 desaturase
New canxfad2.pep = canola delta-12 desaturase
cornfad2e.PRO = corn delta-12 desaturase

Attachment II



New soyxfad.2pep = soybean delta-12 desaturase
CASTOR FA-OH.PEP = castor 12-hydroxylase
New araxfad2.pep = Arabidopsis delat-12 desaturase
New canxfad2.pep = canola delta-12 desaturase
cornfad2e.PRO = corn delta-12 desaturase

Attachment III

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LAGGRM-----SS-----KLESE-K-----NP----- Majority
      10              20              30              40
GGRGRV-----AK-----VEVQGF-----KP----- New soymfad2 pep
GGGGRM-----ST-----VIIS-N-----NS-----EK CASTOR FA-OH PEP
GAGGRMPVPTSS-----KKSE-T-----DT----- New araxfad2 pep
GAGGRMQVSFFS-----KKSE-T-----DN----- New canxfad2 pep
GAGGRM-----TE-----KERE-FQEQLARATGGAA----- cornfad2e.PRO
GAGGRMQVSPSP-----KKSE-T-----DT----- bjunfad2.PRO
GAGE-Y-----TS-----VTNE-N-----NP----- helianfad2.PRO
GAGGRM-----PI-----NPQG-K-----NSEEV----- petroselFad2.PRO
GAGGRM-----SAPNGETEVK-R-----NP----- potatoFad2.PRO
GAGGRV-----TK-----IEAQ-K-----KP----- Peanut.PRO
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-----LQRVPHEKPPFTVGD LKKAIPPHCFKRSLPRSF S Majority
      50              60              70              80
-----LSRVPNNTKPPFTVGD LKKAIPPHCFQRSLLTSE S New soymfad2 pep
GSSSHLERAPHTKPPYTLGNLKR AIPPHCFERSFVRSF S CASTOR FA-OH PEP
-----TKRVPCCKPPFSVGD LKKAIPPHCFKRSLPRSF S New araxfad2 pep
-----IKRVPCETRPFTVGD LKKAIPPHCFKRSLPRSF S New canxfad2 pep
-----MQRSPVEKPPFTLGG I KKAIPPHCFERSVLKSE S cornfad2e.PRO
-----LKRVPCEETRPFTVGD LKKAIPPHCFKRSLPRSF S bjunfad2.PRO
-----LDRVPDHAKPPFTIGD LKKAIPPHCFQRSLTRSF S helianfad2.PRO
-----LQRVPVSKPPFTVGD I KKAIPPHCFKRSLVIRSF S petroselFad2.PRO
-----LQKVPTSKPPFTVGD I KKAIPPHCFQRSLIRSF S potatoFad2.PRO
-----LSRVPHSNPPFSVGD LKKAIPPHCFERSLFI S F S Peanut.PRC
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VYDLIIASCFYVATTYFHL L L L SYIAWPLYWACQG Majority
      90              100              110              120
VYDLSFAF-1FYIATTYFHL L L P Q P F S LIAWPIYWVLQG New soymfad2 pep
FAYNFCLSLFSLYSIA TNFFYI S S PLSYVAWLVLVQLQG CASTOR FA-OH PEP
LISDIIIASCFYVATNYFSL L L P Q P L SYLA W PLYWACQG New araxfad2 pep
LIWDIIIASCFYVATTYFHL L L P L S Y F A W PLYWACQG New canxfad2 pep
VHDLVIAAALLYFALAIIFAL P S P L R Y A A W PLYWIAQG cornfad2e.PRO
LIWDIIIVASCYFVATTYFHL L L P L S Y V A W PLYWACQG bjunfad2.PRO
VLSDLTITAVLYHIA TTYFHL L L P L S S I A W A S Y W V V Q G helianfad2.PRO
VYDVLVIASILYFVATNYIQL L L P H P L S Y I A W P I H G F V Q G petroselFad2.PRO
VYDILILVSIMYYVANTYFHL L L P S P Y C Y I A W P I Y W I C Q G potatoFad2.PRO
VYDILLVAYLLFYIATTYFHL L P P F S F L A W P I Y W A I Q G Peanut.PRO
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LTGVWVIAHECGHHAFSDYQWLDDTVGLILHSSLLVPY Majority
      130              140              150              160
LTGVWVIAHECGHHAFSKYQWVDDTVGLTHSTLLVPY New soymfad2 pep
LTGLWVIGHECGHHAFSEYQLADDIVGLIVH'SALLVPY CASTOR FA-OH PEP
LTGIWVIAHECGHHAFSDYQWLDLDDTVGLIFHSELLVPY New araxfad2 pep
LTGVWVIAHECGHAAPSDYQWLDLDDTVGLIFHSELLVPY New canxfad2 pep
LTGVWVIAHECGHHAFSDY-----LDDTVGLVHHSLLMVPY cornfad2e.PRO
LTGVWVIAHECGHHAFSDYQWLDLDDTVGL-----HSELLVPY bjunfad2.PRO
LTGIWVIAHECGHHAFSDYQWVDDTVGVHHSLLVPY helianfad2.PRO
LTGIWVIAHECGHHAFSDYQWLDLDDTVGLILHSSLLVPY petroselFad2.PRO
LTGIWVNAHECGHHAFSDYQWVDDTVGLILHSA L L V P Y potatoFad2.PRO
LTGVWVIAHECGHHAFSKYQWVDDTVGLILHSSLLVPY Peanut.PRO
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SWKYSHRRHHSNTGSLERDEVFVPPKPKSKVSWYSKYL - N Majority
170 180 190 200
SWKISHRRHHSNTGSLDRDEVFVPPKPKSKVSAWFSKYL - N New soymfad2.pep
SWKYSHRRHHSNTGSLERDEVFVPPKPKSKVSWYSKYL - N CASTOR FA-OH.PEP
SWKYSHRRHHSNTGSLERDEVFVPPKPKSKVSAWKYWKYL - N New araxfad2.pep
SWKYSHRRHHSNTGSLERDEVFVPPKPKSKVSAWFSKYL - N New canxfad2.pep
SWKYSHRRHHSNTGSLERDEVFVPPKPKSKVSAWFSKYL - N cornfad2e.PRO
SWKYSHRRHHSNTGSLERDEVFVPPKPKSKVSAWFSKYL - N bjunfad2.PRO
SWKYSHRRHHSNTGSLERDEVFVPPKPKSKVSAWFSKYL - N helianfad2.PRO
SWKYSHRRHHSNTGSLERDEVFVPPKPKSKVSAWFSKYL - N petroselFad2.PRO
SWKYSHRRHHSNTGSLERDEVFVPPKPKSKVSAWFSKYL - N potatoFad2.PRO
SWKISHRRHHSNTGSLDRNEVFVPPKPKSKVSWYNKYM - N Peanut.PRO

PPGRVVSLLTVTLTLGWPLYLAFNVSGRPYDR - FACHYDP Majority
210 220 230 240
PLGRAVSLLVTLTLGWPLYLAFNVSGRPYDS - FASHYHP New soymfad2.pep
PPGRVLTLLAATLLLGWPLYLAFNVSGRPYDR - FACHYDP CASTOR FA-OH.PEP
PLGRIMMLTVQFTLGLWPLYLAFNVSGRPYDG - FACHFEP New araxfad2.pep
TFGRVMTLVQFTLGLWPLYLAFNVSGRPYDG - FACHFEP New canxfad2.pep
PVGRVHVHVQFTLGLWPLYLAFNVSGRPYPR - FACHFEP cornfad2e.PRO
PLGRVMTLVQFTLGLWPLYLAFNVSGRPYPEGFACHFEP bjunfad2.PRO
TVGRIVSMFVTLTLGLWPLYLAFNVSGRPYDR - FACHYVP helianfad2.PRO
PPGRVLTLLVTLTLGLWPLYLAFNVSGRPYER - FACHYDP petroselFad2.PRO
PPGRVLSLTLTLTLGLWPLYLAFNVSGRPYDR - FACHYDP potatoFad2.PRO
PPGRAISLFTLTLTLGLWPLYLAFNVSGRPYDR - FASHYDP Peanut.PRO

APIYNDRERLQIYVSDAGILAVCYGLYRYAAAKGLAWVV Majority
250 260 270 280
APIYSNRERLLIYVSDVAFSVTYSLYRVATLFGVLVWLL New soymfad2.pep
APIFSEERERLQIYIADLGIFATTFVLYQATMAKGLAWVM CASTOR FA-OH.PEP
APIYNDRERLQIYVSDAGILAVCYGLYRYAAAKGLAWVV New araxfad2.pep
APIYNDRERLQIYVSDAGILAVCYGLLPYAAVQGVASMV New canxfad2.pep
APIYNDRERLQIYVSDAGVVAFAFGLYKLAAAFGVWWVV cornfad2e.PRO
APIYNDRERLQIYVSDAGILAVCYGLYRYAAAKGLAWVV bjunfad2.PRO
APMYNERKRYQIVMSDIGIVITSFILYRVAMAKGLVWVI helianfad2.PRO
APIYSDRERLQIYVSDAGVLAWSYGLYRLAVAKGLTWVL petroselFad2.PRO
APIYNNRERLQIYVSDAGVLAWSYGLYRLAVAKGLTWVL potatoFad2.PRO
APIYSNRERLLIYVSDSVFAVTYLLYHIAATLKGLGWVV Peanut.PRO

YGVPLLLIVNGFLVLITYLQHTHPSLPHYDSSEWDWLRG Majority
290 300 310 320
YGVPLLLIVNGFLVLITYLQHTHPSLPHYDSSEWDWLRG New soymfad2.pep
YGVPLLLIVNGFLVLITYLQHTHPSLPHYDSSEWDWLRG CASTOR FA-OH.PEP
YGVPLLLIVNGFLVLITYLQHTHPSLPHYDSSEWDWLRG New araxfad2.pep
YGVPLLLIVNGFLVLITYLQHTHPSLPHYDSSEWDWLRG New canxfad2.pep
YGVPLLLIVNGFLVLITYLQHTHPSLPHYDSSEWDWLRG cornfad2e.PRO
YGVPLLLIVNGFLVLITYLQHTHPSLPHYDSSEWDWLRG bjunfad2.PRO
YGVPLLLIVNGFLVLITYLQHTHPSLPHYDSSEWDWLRG helianfad2.PRO
YGVPLLLIVNGFLVLITYLQHTHPSLPHYDSSEWDWLRG petroselFad2.PRO
YGVPLLLIVNGFLVLITYLQHTHPSLPHYDSSEWDWLRG potatoFad2.PRO
YGVPLLLIVNGFLVLITYLQHTHPSLPHYDSSEWDWLRG Peanut.PRO

January 26, 1998 5 24 PM

L A T V D R D Y G I L N K V F H N I T D T H V A H H L F S T M P H Y H A M E A	Majority
330 340 350 360	
L A T M D R D Y G I L N K V F H H I T D T H V A H H L F S T M P H Y H A M E A	New soyxfad2.pep
M V T V D R D Y G V L N K V F H N I A D T H V A H H L F A T V P H Y H A M E A	CASTOR FA-OH.PEP
L A T V D R D Y G I L N K V F H N I T D T H V A H H L F S T M P H Y H A M E A	New araxfad2.pep
L A T V D R D Y G I L N Q G F H N I T D T H E A H H L F S T M P H Y H A M E A	New canxfad2.pep
L A T M D R D Y G I L N R V F H N I T D T H V A H H L F S T M P H Y H A M E A	cornfad2e.PRO
L A T V D R D Y G I L N K V F H N I T D T H V A H H L F S T M P H Y H A M E V	bjunfad2.PRO
L A T V D R D Y G V L N K V F H H I T D T H V V H H L F S T M P H Y H A M E A	helianfad2.PRO
L A T V D R D Y G I L N K V L H N I T D T H V A H H L F S T M P H Y H A M E A	petroselFad2.PRO
L A T C D R D Y G V L N K V F H N I T D T H V V H H L F S T M P H Y H A M E A	potatoFad2.PRO
L A T V D R D Y G I L N K A F H H I T D T H V A H H L F S T M P H Y H A M E A	Peanut.PRO

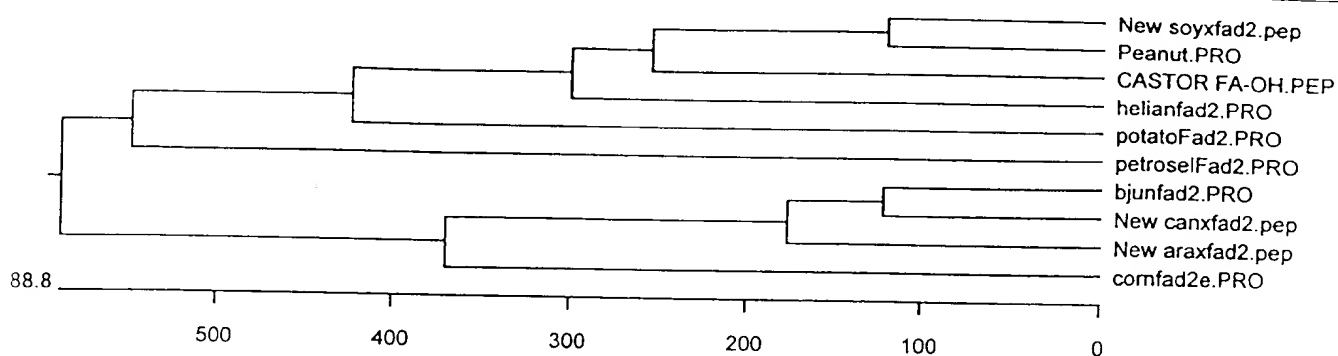
K A I K P I L G D Y Y Q F D G T P F Y K A M W P E A K E C L Y V E P D E G G S	Majority
370 380 390 400	
N A I K P I L G E Y Y Q F D D T P F Y K A L W R E A R E C L Y V E P D E G T S	New soyxfad2.pep
K A I K P I M G E Y Y R Y D G T P F Y K A L W R E A K E C L F V E P D E G A P	CASTOR FA-OH.PEP
K A I K P I L G D Y Y Q F D G T P W Y V A M X R E A K E C I Y V E P D R E G D	New araxfad2.pep
K A I K P I L G E Y Y Q F D G T P V V K A M W R E A K E C L Y V E P D R Q G E	New canxfad2.pep
K A I R P I L G D Y Y H F D P T P V A K A T W R E A G E C I Y V E P E D - - -	cornfad2e.PRO
K A I K P I L G D Y Y Q F D G T P W V K A M W R E A K E C I Y V E P D R Q G E	bjunfad2.PRO
K A L E P V L G E Y Y R E D K T P F Y V A M W R E M K E C L F V E Q D D E G K	helianfad2.PRO
K A I K P I L G D Y Y R F D D T P V V K A M W R E A K E C L Y V E P D E G D Q	petroselFad2.PRO
K A V K P L L G D Y Y Q F D G T P I Y K E M W R E A K E C L Y V E K D E S S Q	potatoFad2.PRO
N A I K P I L G D Y Y Q F D G T P F Y K A L W R E A K E C L Y V E P D D G A S	Peanut.PRO

K G V F W Y N N K L - - - - -	Majority
410	
K G V Y W Y N K Y	New soyxfad2.pep
Q G V F W Y N K Y	CASTOR FA-OH.PEP
K G V Y W Y N N K L	New araxfad2.pep
K G V F W Y N N	New canxfad2.pep
K G V F W Y N K K F	cornfad2e.PRO
K G V F W Y N N K L	bjunfad2.PRO
- G V F W Y F N R M - - - -	helianfad2.PRO
K G I F W Y N N K L - - - -	petroselFad2.PRO
K G V F W Y N K L - - -	potatoFad2.PRO
K G V Y W Y N K F	Peanut.PRO

ation: Separation #2: Shade (with bright turquoise at 40% fill) residues that match araxfad2.pep exactly.

New soyxfad2.pep = soybean delta-12 desaturase
 CASTOR FA-OH.PEP = castor 12-hydroxylase
 New araxfad2.pep = Arabidopsis delta-12 desaturase
 New canxfad2.pep = canola delta-12 desaturase
 cornfad2e.PRO = corn delta-12 desaturase
 bjunfad2.PRO = Brassica juncea delta-12 desaturase
 helianfad2.PRO = sunflower delta-12 desaturase
 petroselFad2.PRO = parsley delta-12 desaturase
 potatoFad2.PRO = potato delta-12 desaturase

Attachment IV



New soylfad2.pep = soybean delta-12 desaturase
 CASTOR FA-OH.PEP = castor 12-hydroxylase
 New araxfad2.pep = *Arabidopsis* delta-12 desaturase
 New canxfad2.pep = canola delta-12 desaturase
 cornfad2e.PRO = corn delta-12 desaturase
 bjunfad2.PRO = *Brassica juncea* delta-12 desaturase
 helianfad2.PRO = sunflower delta-12 desaturase
 petroselFad2 PRO = parsley delta-12 desaturase
 potatoFad2.PRO = potato delta-12 desaturase

Attachment V

	50	60	70	80	Majority
- - - - -	-	-	-	-	New araxfad3.pep
- - - - -	-	-	L S S S S Y	- - -	New araxfadd.pep
- - - - -	-	-	- - - - -	- - -	New canxfad3.pep
- - - - -	-	-	P S S P R F	- - -	New canxfadd.pep
- - - - -	-	-	- - - - -	- - -	New soxsfad3.pep
K E L A P V I P R P R T G A A L S S T S R V E F L D T N K V V A G P K F Q P L R	-	-	- - - - -	- - -	New soxsfadd.pep
- - - - -	-	-	- - - - -	- - -	New soxsfad2.pep
- - - - -	-	-	- - - - -	- - -	CASIOR FA-OH.PEP
- - - - -	-	-	- - - - -	- - -	CORN FAD2.PEP
- - - - -	-	-	- - - - -	- - -	New araxfad2.pep
- - - - -	-	-	- - - - -	- - -	New canxfad2.pep
- - - - -	-	-	- - - - -	- - -	lesqOH.pro
- - - - -	-	-	- - - - -	- - -	bjunfad2.PRO
- - - - -	-	-	- - - - -	- - -	helianfad2.PRO
- - - - -	-	-	- - - - -	- - -	petroselFad2.PRO
- - - - -	-	-	- - - - -	- - -	potatoFad2.PRO

New soyxfad2.pep = soybean delta-12 desaturase
 CASTOR FA-OH.PEP = castor 12-hydroxylase
 New araxfad2.pep = Arabidopsis delta-12 desaturase
 New canxfad2.pep = canola delta-12 desaturase
 CORN FAD2.PEP = corn delta-12 desaturase
 bjunfad2.PRO = Brassica juncea delta-12 desaturase
 helianfad2.PRO = sunflower delta-12 desaturase
 petroselFad2.PRO = parsley delta-12 desaturase
 potatoFad2.PRO = potato delta-12 desaturase
 Lesqui = Lesquerella fendleri 12-hydroxylase
 New araxfad1d.pep = Arabidopsis plastidic delta-15 desaturase
 New canxfad1d.pep = canola plastidic delta-15 desaturase
 New soyxfad1d = soybean plastidic delta-15 desaturase
 New soyxfad1 = soybean microsomal delta-15 desaturase
 New araxfad3.pep = Arabidopsis microsomal delta-15 desaturase
 New canxfad1d.pep = canola microsomal delta-15 desaturase

				Majority
90	100	110	120	
-----				New araxfad3.pep
K T S S S P L S F G L N S R D G F T R N W A L -----				New araxfadd.pep
-----				New canxfad3.pep
R L N ----- S R N W A L -----				New canxfadd.pep
-----				New soxxfad3.pep
N L R ----- E R N W G L -----				New soxxfadd.pep
-----				New soxxfad2.pep
-----				CASTOR FA-OH.PEP
----- P L P P P C K S C R H H R S F F -----				CORN FAD2.PEP
-----				New araxfad2.pep
-----				New canxfad2.pep
-----				lesqOH.pro
-----				bjunfad2.PRO
-----				helianfad2.PRO
-----				petroselFad2.PRO
-----				potatoFad2.PRO

				G A G Majority
130	140	150	160	
-----				New araxfad3.pep
----- N V S T P L T T -----				New araxfadd.pep
-----				New canxfad3.pep
----- N V T T P L T V -----				New canxfadd.pep
----- K D T K P L A Y -----				New soxxfad3.pep
----- K V S A P L R V -----				New soxxfadd.pep
-----				New soxxfad2.pep
-----				CASTOR FA-OH.PEP
P G Q E K R G E R G E A R C P P D L L C P D A A V T T S S V S V R S K M -----				CORN FAD2.PEP
-----				New araxfad2.pep
-----				New canxfad2.pep
-----				lesqOH.pro
-----				bjunfad2.PRO
-----				helianfad2.PRO
-----				petroselFad2.PRO
-----				potatoFad2.PRO

IRMAVSPSSK-----KS-----E-----T Majority															
170				180				190				200			
--VAMDQRTN-----VN-----GDPGAGDRKKE															
--PIFEEELPL-----EE-----D-----N															
--VAMDQRN-----AN-----G-----D															
--DSSSSPPI-----EE-----S-----P															
--AANNGYQQ-----EG-----S-----S															
--ASIEEEQKSVDL-----TN-----G-----S															
GRVAKV-----E-----VQ-----G-----K															
GRNSTV-----I-----INNSEKKGG-----S															
GRMTEKERER-----QEQLARAT-----G-----G															
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GRMTEKERER-----QEQLARAT-----G-----G															
GRMTEKERER-----QEQLARAT-----G-----G															

FLRSFSYVVRDLIIIVSALY-VATTYFPL--LP-PLS--A Majority

250	260	270	280	
FLRSFSYVVRDLIIIVSALY-VATTYFPL--LP-PLS--A				Majority
FLRSFSYVVRDLIIIVSALY-VATTYFPL--LP-PLS--A				New araxfad3.pep
FLRSFSYVVRDLIIIVSALY-VATTYFPL--LP-PLS--A				New araxfad2.pep
FLRSFSYVVRDLIIIVSALY-VATTYFPL--LP-PLS--A				New canxfad3.pep
FLRSFSYVVRDLIIIVSALY-VATTYFPL--LP-PLS--A				New canxfad2.pep
FLRSFSYVVRDLIIIVSALY-VATTYFPL--LP-PLS--A				New soyxfad3.pep
FLRSFSYVVRDLIIIVSALY-VATTYFPL--LP-PLS--A				New soyxfad2.pep
FLRSFSYVVRDLIIIVSALY-VATTYFPL--LP-PLS--A				CASTOR FA-OH.PEP
FLRSFSYVVRDLIIIVSALY-VATTYFPL--LP-PLS--A				CORN FAD2.PEP
FLRSFSYVVRDLIIIVSALY-VATTYFPL--LP-PLS--A				New araxfad2.pep
FLRSFSYVVRDLIIIVSALY-VATTYFPL--LP-PLS--A				New canxfad2.pep
FLRSFSYVVRDLIIIVSALY-VATTYFPL--LP-PLS--A				lesqOH.pro
FLRSFSYVVRDLIIIVSALY-VATTYFPL--LP-PLS--A				bjunfad2.PRO
FLRSFSYVVRDLIIIVSALY-VATTYFPL--LP-PLS--A				helianfad2.PRO
FLRSFSYVVRDLIIIVSALY-VATTYFPL--LP-PLS--A				petroselFad2.PRO
FLRSFSYVVRDLIIIVSALY-VATTYFPL--LP-PLS--A				potatoFad2.PRO

WPLYWAAQGCVLGTGIWVIGHECGHHAFFSDYQWLD DDTVGLI Majority

290	300	310	320	
WPLYWAAQGCVLGTGIWVIGHECGHHAFFSDYQWLD DDTVGLI				Majority
WPLYWAAQGCVLGTGIWVIGHECGHHAFFSDYQWLD DDTVGLI				New araxfad3.pep
WPLYWAAQGCVLGTGIWVIGHECGHHAFFSDYQWLD DDTVGLI				New araxfad2.pep
WPLYWAAQGCVLGTGIWVIGHECGHHAFFSDYQWLD DDTVGLI				New canxfad3.pep
WPLYWAAQGCVLGTGIWVIGHECGHHAFFSDYQWLD DDTVGLI				New canxfad2.pep
WPLYWAAQGCVLGTGIWVIGHECGHHAFFSDYQWLD DDTVGLI				New soyxfad3.pep
WPLYWAAQGCVLGTGIWVIGHECGHHAFFSDYQWLD DDTVGLI				New soyxfad2.pep
WPLYWAAQGCVLGTGIWVIGHECGHHAFFSDYQWLD DDTVGLI				CASTOR FA-OH.PEP
WPLYWAAQGCVLGTGIWVIGHECGHHAFFSDYQWLD DDTVGLI				CORN FAD2.PEP
WPLYWAAQGCVLGTGIWVIGHECGHHAFFSDYQWLD DDTVGLI				New araxfad2.pep
WPLYWAAQGCVLGTGIWVIGHECGHHAFFSDYQWLD DDTVGLI				New canxfad2.pep
WPLYWAAQGCVLGTGIWVIGHECGHHAFFSDYQWLD DDTVGLI				lesqOH.pro
WPLYWAAQGCVLGTGIWVIGHECGHHAFFSDYQWLD DDTVGLI				bjunfad2.PRO
WPLYWAAQGCVLGTGIWVIGHECGHHAFFSDYQWLD DDTVGLI				helianfad2.PRO
WPLYWAAQGCVLGTGIWVIGHECGHHAFFSDYQWLD DDTVGLI				petroselFad2.PRO
WPLYWAAQGCVLGTGIWVIGHECGHHAFFSDYQWLD DDTVGLI				potatoFad2.PRO

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L H S S L L V P Y F S W K Y S H R	H H S N T G S L E R D E V F V P K P K	A - Majority
330	340	350 360
L H S T I L V P Y H G W F I S H R T H R	Q N H G H V E N D E S W A L P E R V	New araxfad3.pep
L H S S I L V P Y H G W F I S H R T H R	Q N H G H V E N D E S W H M S E K I	New araxfadd.pep
L H S T I L V P Y H G W F I S H R T H R	Q N H G H V E N D E S W A L P E K L	New canxfad3.pep
L H S S I L V P Y H G W F I S H R T H R	Q N H G H V E N D E S W H M S E K I	New canxfadd.pep
L H S S I L V P Y H G W R I S H R T H R	Q N H G H I E K D E S W A L T E K I	New soyxfad3.pep
L H S S I L V P Y H G W R I S H R T H R	Q H H G H A E N D E S W H L P E K L	New soyxfadd.pep
L H S T I L V P Y F S W A I S H R T H R	G S L D F R A P K V	New soyxfad2.pep
V H S A L T V P Y F S W K Y S H R T H R	I G S L E R D E V F V P K K	CASTOR FA-OH.PEP
L H S S I L V P Y F S W K Y S H R T H R	I G S L E R D E V F V P K E L	CORN FAD2.PEP
F H S K T L V P Y F S W A I S H R T H R	I G S L E R D E V F V P K K	New araxfad2.pep
F H S S I L V P Y F S W A I S H R T H R	I G S L E R D E V F V P K K	New canxfad2.pep
F H S S I L V P Y F S W A I S H R T H R	I G S L E R D E V F V P K K	lesqOH.pro
F H S S I L V P Y F S W K Y S H R T H R	I G S L E R D E V F V P K K	bjunfad2.PRO
L H S S I L V P Y F S W K Y S H R T H R	I G S L E R D E V F V P K K	helianfad2.PRO
L H S S I L V P Y F S W K Y S H R T H R	I G S L E R D E V F V P K K	petroselFad2.PRO
L H S A L L V P Y F S W K Y S H R T H R	I G S L E R D E V F V P K K	potatoFad2.PRO

- W Y S K Y L - N N P T G R V L P L T V P L T - L G W P L Y L A F N V S G R P Y	Majority
370	380 390 400
- - Y K K - L - P H S T - R M L P Y T - P L P M L A Y P F M A C Y R S P	KE - New araxfad3.pep
- - Y N T - L - D K P T - R F F F F F L P L A M L A Y P F M A W A R S P	G K K - New araxfadd.pep
- - Y K N - L - S H S T - R M L P Y T - P L P M L A Y P F M A W Y R S P	G K E - New canxfad3.pep
- - Y K S - L - D K P T - R F F F F F L P L A M L A Y P F M A W A R S P	G K K - New canxfadd.pep
- - Y K N - L - D S M T - R L I R F T V P P L F V Y P I Y F S R S P	G K E - New soyxfad3.pep
- - F F S - L - D T V T - R M L P F T A P E P L L A F P V Y F S R S P	G K T - New soyxfadd.pep
A W F S K Y L - N N P T G R V L P L T V P L T - L G W P L Y L A F N V S G R P Y	New soyxfad2.pep
A W Y C K Y L - N N P P G R V L T L A A T L L - L G W P L Y L A F N V S G R P Y	CASTOR FA OH.PEP
P W Y T P Y V Y N N P P G R V L T L A A T L L - L G W P L Y L A F N V S G R P Y	CORN FAD2.PEP
K W Y G K Y L - N N P P G R V L T L A A T L L - L G W P L Y L A F N V S G R P Y	New araxfad2.pep
S - G T A S T - S T T F E A T V M L T V P L T - L G W P L Y L A F N V S G R P Y	New canxfad2.pep
K W Y V K Y L - N N P P G R V L T L A A T L L - L G W P L Y L A F N V S G R P Y	lesqOH.pro
K W Y G K Y L - N N P P G R V L T V M L T V P L T - L G W P L Y L A F N V S G R P Y	bjunfad2.PRO
P W Y S K Y F - N N T V G R V L T L A A T L L - L G W P L Y L A F N V S G R P Y	helianfad2.PRO
Q S V A K Y T - N N P P G R V L T L A A T L L - L G W P L Y L A F N V S G R P Y	petroselFad2.PRO
G W Y S K Y L - N N P P G R V L S L T I T L T - L G W P L Y L A F N V S G R P Y	potatoFad2.PRO

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D - - F A S H F D P Y S P I F N D . E R L Q I Y I S D A - - G I L A V C L L Y Majority
      410              420              430              440
- - - - G S H F N P Y S S L F A P S T K L E A T T T C W S T M F S L I - - New araxfad3.pep
- - - - G S H Y H P D S L F L P K E K D V L T S T A C W T A M A L L V - - New araxfadd.pep
- - - - G S H Y N P Y S S L F A P S T K L E A T T T C W S T M L A T L V - - New canxfad3.pep
- - - - G S H Y H P D S L F L P K E K D V L T S T A C W T A M A L L V - - New canxfadd.pep
- - - - G S H F N P Y S N L F P P S E T K G T A I S T L C W A T M F S L I - - New soyxfad3.pep
- - - - G S H F D P S S L F V P N E K D V I T S T A C W A A M L G L L V - - New soyxfadd.pep
D - S F A S H Y H P Y A P I S N E L L V V S D V - - A L F S T Y S New soyxfad2.pep
D - P F A C H Y D P Y G P I F S E L L I A D L - - G L F A T T E V CASTOR FA-OH.PEP
F - P F A C H F D P Y G P I F S E L L I A D L - - G V V A A F A CORN FAD2.PEP
D - S F A C H F P P A P I S N E L L V V S D V - - G I L A V C L L Y New araxfad2.pep
D G G F A C H H P A P I S N E L L V V S D V - - G L F A T T E V New canxfad2.pep
D - G E A S H F P H A P I F K L L I A D L - - G L F A T T E V lesqOH.pro
F E S F A C H F H P A P I S N E L L V V S D V - - G I L A V C L L Y bjunfad2.PRO
D - P F A C H Y V P T S M I E K Y L V M E D I - - G L V I T S F I helianfad2.PRO
E - R F A C H Y D P K S P I S L L A L L V S D A - - G V L A S Y petroselFad2.PRO
D - R F A C H Y D P Y G P I F S E L L I A D L - - G V V A A F A potatoFad2.PRO

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R L A F A K G L A W V L C V Y G V P L L I V N M F L V L I T Y L Q H - - T H P S Majority
      450              460              470              480
A L S F V F G P L A V L F V Y I F V M W D A V Y L H H H G H D E K New araxfad3.pep
C L N F T I G P I Q M L F L G I Y W N V M W D F V Y L H H H G H E D K New araxfadd.pep
Y L S F L V G P V T V L F V G V P Y I F V M W D A V Y L H H H G H D D K New canxfad3.pep
C L N F V M G P M Q M L F L G I Y W N V M W D F V Y L H H H G H E D K New canxfadd.pep
Y L S F I T S P L L V L F L G I Y W F V M W D F V Y L H H H G H H Q K New soyxfad3.pep
G L G F V M G P I Q L L F L G I Y V F V M W D L V T Y L H H H G H E D K New soyxfadd.pep
R V A T L F G L V W L L C V G P I Y W N V M W D F V Y L H H H G H E D K New soyxfad2.pep
D A T M A Y G L A W V M E T G L I A V N C P L A M S Y L Q H - - T H P A CASTOR FA OH.PEP
K L A A A F G V W W V F V A L L A W A V A V A D O H - - T H P CORN FAD2.PEP
R V A A A Q G M A S M I C L A G P I Y W N V M W D F V Y L H H H G H E D K New araxfad2.pep
P R A A V O G V A S S I V F L R P I Y W N V M W D F V Y L H H H G H E D K New canxfad2.pep
R V A A S O G L T A M L E V P I Y W N V M W D F V Y L H H H G H E D K lesqOH.pro
R V A A A Q G V A S M V D I A G P I Y W N V M W D F V Y L H H H G H E D K bjunfad2.PRO
R V A M A Y G L V W V F G V G P I Y W N V M W D F V Y L H H H G H E D K helianfad2.PRO
R L A V A F G L T W V L C V G G P I Y W N V M W D F V Y L H H H G H E D K petroselFad2.PRO
R I A L V K G L A W L V C V G P I Y W N V M W D F V Y L H H H G H E D K potatoFad2.PRO

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[illegible]

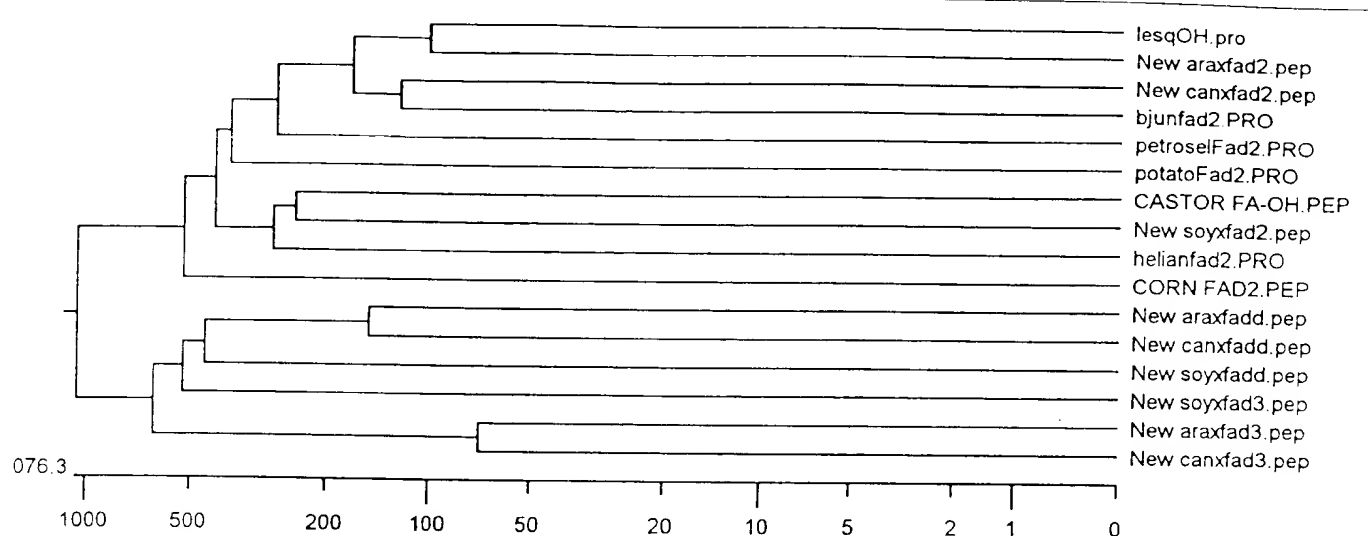
ay, January 26, 1998 5:42 PM

G T P V Y K A M W - - - - R E A R E C V Y V E P D - - - - - S - - - - - Majority									
570					580				
590					600				
ESLVASIKKDHVSDTGDIVFY	T	PDLYVYAS	-	-	-	-	-	-	New araxfad3.pep
EILAKSIFEDHIVSDEGEV	V	YKA	-	-	-	-	-	-	New araxfadd.pep
ESLVASIFKDHVSDTGDIVFY	T	PDLYVYAS	-	-	-	-	-	-	New canxfad3.pep
GILAKSIFEDHIVSDEGDVV	Y	A	-	-	-	-	-	-	New canxfadd.pep
KYLIQSMFQDHFVSDTGDIV	Y	QT	-	-	-	-	-	-	New soyxfad3.pep
GEIIPSFFTDFHFVSDTGDIV	Y	QT	-	-	-	-	-	-	New soyxfadd.pep
DDTFEYKALW	-	-	-	-	-	-	-	-	New soyxfad2.pep
GTTFEYKALW	-	-	-	-	-	-	-	-	CASTOR FA-OH.PEP
PTTFVAKATW	-	-	-	-	-	-	-	-	CORN FAD2.PEP
GTTFEYKALW	-	-	-	-	-	-	-	-	New araxfad2.pep
GTTFEYKALW	-	-	-	-	-	-	-	-	New canxfad2.pep
GTTFEYKALW	-	-	-	-	-	-	-	-	lesqOH.pro
GTTFEYKALW	-	-	-	-	-	-	-	-	bjunfad2.PRO
KTFEYKALW	-	-	-	-	-	-	-	-	helianfad2.PRO
DTTFEYKALW	-	-	-	-	-	-	-	-	petroselFad2.PRO
GTTFEYKALW	-	-	-	-	-	-	-	-	potatoFad2.PRO

- G - - - - - K G V F W Y N N K L - - - Majority									
610					620				
- D - - - - -	-	-	-	-	-	-	-	-	New araxfad3.pep
- N L Y G E V	-	-	-	-	-	-	-	-	New araxfadd.pep
- D - - - - -	-	-	-	-	-	-	-	-	New canxfad3.pep
- N L Y G E I	-	-	-	-	-	-	-	-	New canxfadd.pep
- L - - - - -	-	-	-	-	-	-	-	-	New soyxfad3.pep
- G - - - - -	-	-	-	-	-	-	-	-	New soyxfadd.pep
S E - - - - -	-	-	-	-	-	-	-	-	New soyxfad2.pep
P T - - - - -	-	-	-	-	-	-	-	-	CASTOR FA-OH.PEP
- R - - - - -	-	-	-	-	-	-	-	-	CORN FAD2.PEP
D E - - - - -	-	-	-	-	-	-	-	-	New araxfad2.pep
E K - - - - -	-	-	-	-	-	-	-	-	New canxfad2.pep
G K - - - - -	-	-	-	-	-	-	-	-	lesqOH.pro
E K - - - - -	-	-	-	-	-	-	-	-	bjunfad2.PRO
K G - - - - -	-	-	-	-	-	-	-	-	helianfad2.PRO
Q G - - - - -	-	-	-	-	-	-	-	-	petroselFad2.PRO
Q G - - - - -	-	-	-	-	-	-	-	-	potatoFad2.PRO

ation 'Decoration #1': Shade (with bright turquoise at 40% fill) residues that match araxfad2.pep exactly.

Attachment VI



New soylfad2.pep = soybean delta-12 desaturase
 CASTOR FA-OH.PEP = castor 12-hydroxylase
 New araxfad2.pep = *Arabidopsis* delta-12 desaturase
 New canxfad2.pep = canola delta-12 desaturase
 CORN FAD2.PEP = corn delta-12 desaturase
 bjunfad2.PRO = *Brassica juncea* delta-12 desaturase
 helianfad2.PRO = sunflower delta-12 desaturase
 petroselFad2.PRO = parsley delta-12 desaturase
 potatoFad2.PRO = potato delta-12 desaturase
 lesqOH = *Lesquerella fendleri* 12-hydroxylase
 New araxfadd.pep = *Arabidopsis* plastidic delta-15 desaturase
 New canxfadd.pep = canola plastidic delta-15 desaturase
 New soylfadd = soybean plastidic delta-15 desaturase
 New soylfad3 = soybean microsomal delta-15 desaturase
 New araxfad3.pep = *Arabidopsis* microsomal delta-15 desaturase
 New canxfad3.pep = canola microsomal delta-15 desaturase

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Edited by

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and

Nora Wan Lem



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EXPRESSION OF CASTOR AND *L. FENDLERI* OLEATE 12-HYDROXYLASES IN TRANSGENIC PLANTS

Effects on lipid metabolism and inferences on structure-function relationships in fatty acid hydroxylases

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Introduction

Ricinoleic acid (D-12-hydroxyoctadec-cis-9-enoic acid), is an hydroxylated fatty acid which constitutes 85-90% of the seed fatty acids in castor bean plants (*Ricinus communis* L.). This unusual fatty acid is also one of a series of related Hydroxy Fatty Acids (HFAs) produced in the seeds of *Lesquerella* species. In these species, which, like *A. thaliana* and rapeseed belong to the *Brassicaceae* family, ricinoleic acid is generally a minor constituent. Major HFAs include densipolic (12-OH, 18:2 (3,9)), lesquerolic (14-OH, 20:1 (9)) and auricolic (14-OH, 20:2 (3,9)) acids.

In castor, where metabolism of HFAs has been studied in most detail, ricinoleic acid is synthesized in seeds on phosphatidyl choline, then very efficiently removed from membranes and transferred to the triacylglycerol pool (Bafor et al., 1991).

Recently, we have reported the isolation of a cDNA clone encoding the oleate 12- hydroxylase from castor (van de Loo et al., 1995). Constitutive expression of the hydroxylase cDNA in transgenic tobacco resulted in accumulation of low levels of ricinoleate in seed lipids, but not in leaves and roots. In order to further characterize metabolism of HFAs in transgenic plants, we have introduced this cDNA into *A. thaliana*. We report here how lipid metabolism is affected in transgenic plants.

Extrplastidial α -6 desaturases and castor oleate 12- hydroxylase share a number of biochemical characteristics. Cloning of a cDNA encoding the castor hydroxylase has also confirmed that the two enzymes are closely related (van de Loo et al., 1995). Although reaction mechanisms are expected to be similar, they lead to a different outcome. In order to investigate what structural components in these enzymes are responsible for the different outcomes, we have selected to isolate the gene encoding another oleate 12-hydroxylase, from *Lesquerella fendleri*. Multiple comparisons of desaturase and hydroxylase sequences revealed key differences between the two categories of enzymes. We report here investigation of their functional significance.

1. Expression of Castor and *L. fendleri* Hydroxylase Genes in transgenic *A. thaliana*

We designed degenerate primers based on the sequence of castor fatty acid hydroxylase CFah12 and used them to PCR-amplify cDNAs from *L. fendleri*. One such cDNA detected an abundant seed specific transcript on Northern blots of *L. fendleri* RNA. Its sequence had extensive similarity with the CFah12 gene. This cDNA was used to isolate a genomic clone which was introduced into *A. thaliana*.

Expression of the *L. fendleri* gene resulted in accumulation of HFAs in transgenic plants, up to 15% of seed fatty acids, thus establishing the gene encodes *L. fendleri* hydroxylase LFah12.

Transgenic plants expressing CFah12 under the control of a strong seed specific promoter were also obtained. In these plants, HFAs constitute up to 20% of the seed fatty acids. Seed fatty acid composition of *A. thaliana* plants expressing CFah12 or LFah12 is very similar, suggesting the two enzymes have comparable activities in transgenic plants.

Ricinoleic acid is only one of four HFAs produced in transgenic seeds, which also accumulate densipolic, lesquerolic and a small amount of auricolic acid. This suggests that *Arabidopsis* and related *Lesquerella* species metabolize ricinoleic acid in a similar way.

Expression of LFah12 under the control of the CaMV 35S promoter did not affect fatty acid composition of vegetative organs, even though hydroxylase activity could be detected. This implies either poor enzyme activity or efficient turnover of HFAs in non-seed tissues.

Accumulation of HFAs was accompanied with an increase in oleate levels and a concurrent decrease in 18:2 and 18:3, suggesting the oleate 12- desaturase is inhibited in transgenic plants expressing either gene.

2. Investigations of Structure-Function Relationships in Hydroxylases and related Desaturases

We performed multiple comparisons between oleate 12- desaturases, CFah12 and LFah12, and identified six residues conserved among desaturases which differ in fatty acid hydroxylases. Using appropriate growth conditions (Covello and Reed, 1996), we were able to express LFah12 in yeast, under the control of the *GALI* promoter. Yeast strains over-expressing the *Lesquerella* gene accumulated a small amount of ricinoleic acid. We could also detect small levels of 18:2, indicative of some desaturase activity of the enzyme in this context.

In order to establish the functional significance of observed residue differences between desaturases and hydroxylases, we used site-directed mutagenesis to substitute desaturase residues for the corresponding residues in LFah12 at all six positions. In yeast strains expressing the mutant hydroxylase, ratios of 18:2 to ricinoleic acid levels were more than 20 fold higher than in control strains expressing the wild type gene (Fig. 1). This result indicates that these residues are essential in LFah12 in determining the outcome of fatty acid oxidation.

Conclusion

We described here the isolation of a novel fatty acid hydroxylase from *L. fendleri*. We also presented some results from the analysis of transgenic *A. thaliana* plants expressing the castor and *L. fendleri* genes. We plan to use these transgenic plants to dissect mechanisms involved in removing HFAs from membranes, channeling them to storage lipids or breaking them down. We also hope to gain understanding of what controls such mechanisms.

We also reported here the critical role played by a small number of residues in controlling the outcome of fatty acid oxidation. Narrowing down on fewer residues will make it easier to rationalize structural differences between hydroxylases and desaturases, and understand how these differences affect reaction mechanisms.

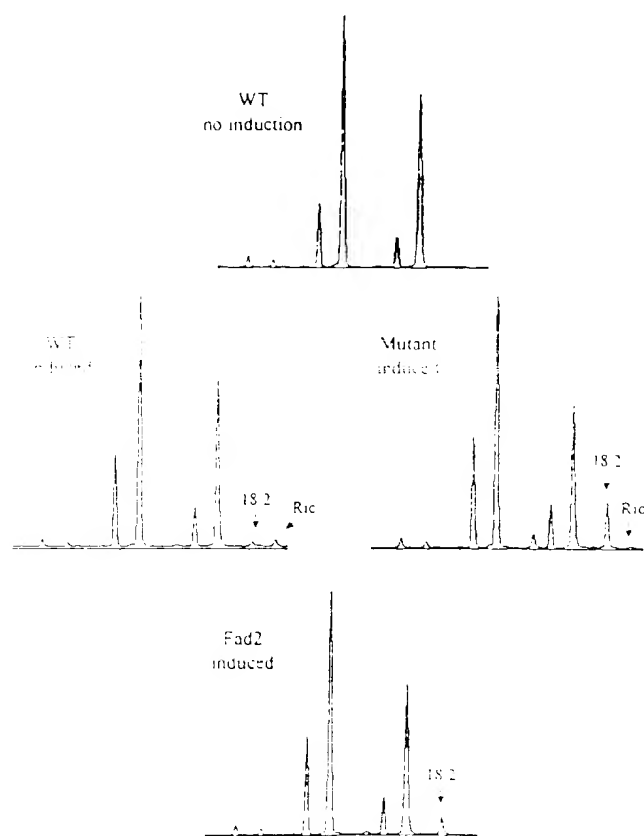
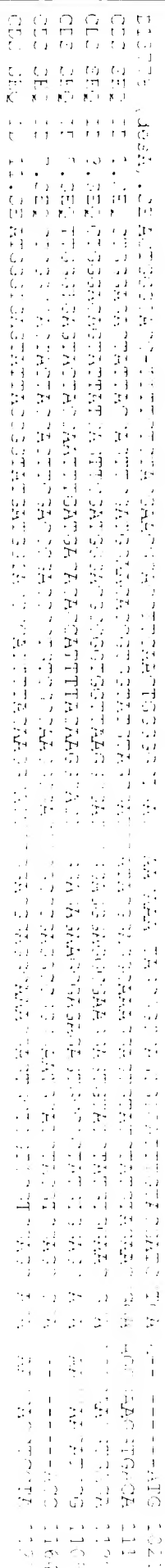
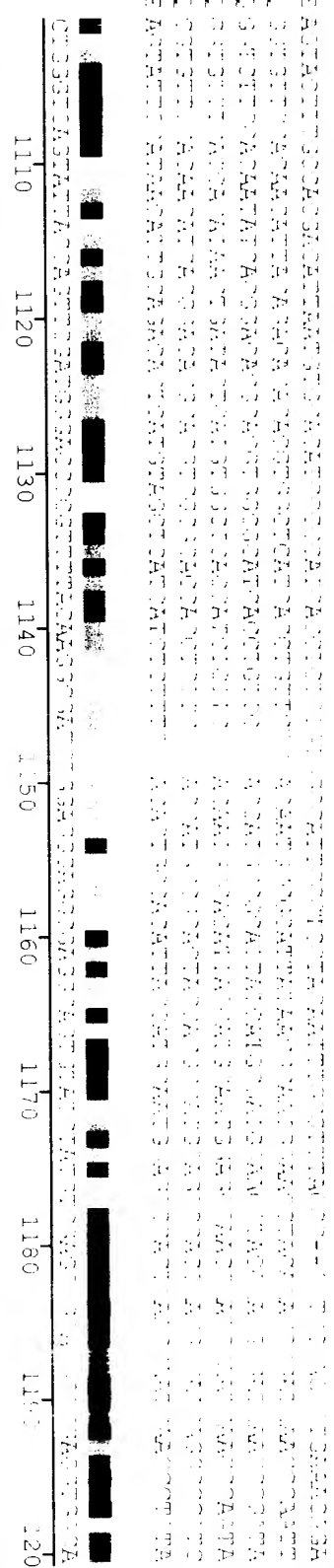
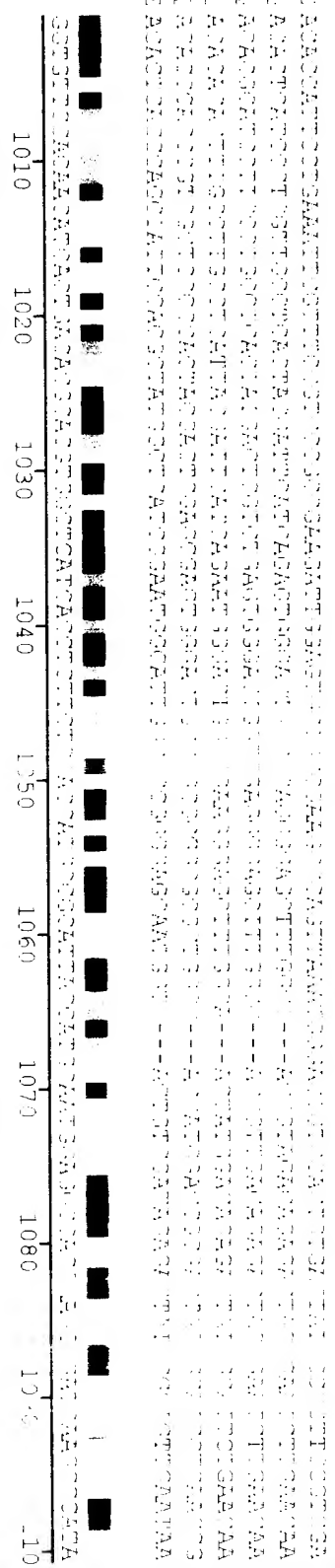
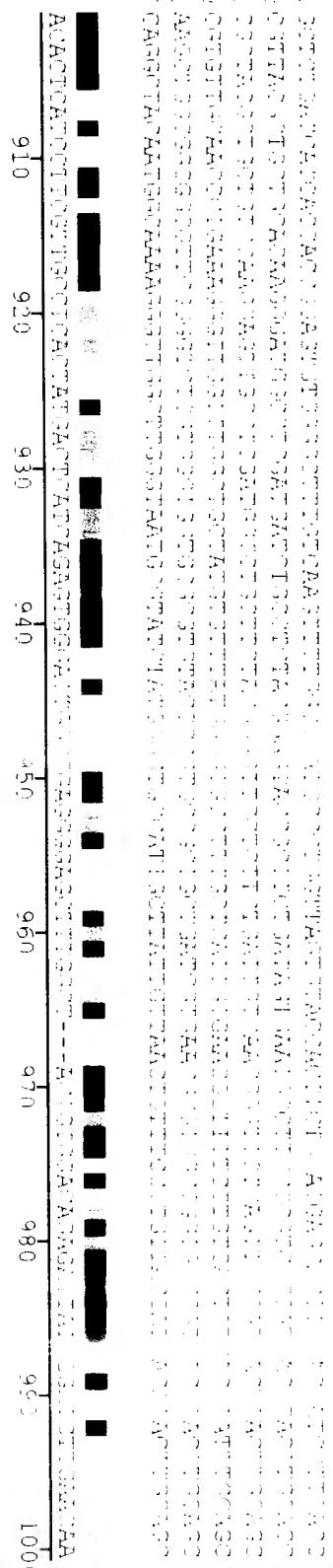


Figure 1: Gas chromatogram of fatty acid methyl esters from yeast strains expressing a wild-type or a mutant fatty acid hydroxylase from *L. fendleri*.

Bator, M., Smith, M.A., Jonsson, L., Stobart, K. and Stymne, S. (1991) Ricinoleic acid biosynthesis and accumulation in microsomal preparations from developing castor-bean (*Ricinus communis*) endosperm. *Biochem. J.* **280**, 507-514.

Coxford, E.S. and Reed, D.W. (1996) Functional expression of the extraplastidial *Arabidopsis thaliana* oleate desaturase gene (*FAD2*) in *Saccharomyces cerevisiae*. *Plant Physiol.* **111**, 223-226.

van de Pelt, L., Broom, P., Turner, S., Somerville, C.R. (1995) An oleate 12-hydroxylase from castor (*Ricinus communis*) is a fatty acyl desaturase homologue. *Proc. Natl. Acad. Sci. USA* **92**, 6743-6747.



1210	1220	1230
------	------	------

D13778 (desA). SEGGTAAGACACTTAACTTCCTCAAA-----TAA	103
CDS SEQ ID 1.SFGAGAAAGGTGTGTATGGTAAATAAATAATTATGA	113
CDS SEQ ID 4.SEQAGAAAGGTGTGTGTGTGTAAATAAATAATTATGA	114
CDS SEQ ID 5.SEQAGAAAGGTGTGTGTGTGTAAATAAATAATTATGA	115
CDS SEQ ID 6.SEQAGAAAGGTGTGTGTGTGTAAATAAATAATTATGA	116
CDS SEQ ID 7.SFGGAAAGGTGTGTGTGTGTAAATAAATAATTATGA	117
CDS SEQ ID 11.SEGCAAAAGGTGTGTGTGTGTAAATAAATAATTATGA	118

Percent Identity

	1	2	3	4	5	6	
1		39.6	40.8	38.8	45.8	39.8	1
2	100.0		85.7	66.0	63.5	65.3	2
3	100.0	16.0		65.8	67.5	64.8	3
4	100.0	46.1	46.6		59.9	70.6	4
5	97.8	50.4	42.7	57.8		58.9	5
6	100.0	47.6	48.6	38.4	60.3		6
	1	2	3	4	5	6	

Divergence

D13778 (desA).SEQ
 CDS SEQ ID 1.SEQ
 CDS SEQ ID 3.SEQ
 CDS SEQ ID 5.SEQ
 CDS SEQ ID 7.SEQ
 CDS SEQ ID 11.SEQ

Percent Identity

	1	2	3	4	5	6	
1		18.5	17.9	17.1	19.7	18.2	1
2	197.7		82.8	68.1	67.9	65.3	2
3	195.0	17.3		63.6	64.8	60.1	3
4	203.0	39.0	43.6		62.5	64.9	4
5	184.8	37.0	40.2	45.2		58.9	5
6	200.0	45.0	51.5	42.9	53.1		6
	1	2	3	4	5	6	

desA.PRO
 SEQ ID 2.PRO
 SEQ ID 4.PRO
 SEQ ID 6.PRO
 SEQ ID 8.PRO
 SED ID 12.PRO

Divergence

